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(57) Abstract

Novel liposomes have a mean diameter not exceeding 200 nm and containing at least 33 mg of one or more entrapped substances per g liposome-forming material. They may also comprise, co-entrapped or covalently linked in the same liposome, a synthetic peptide and an adjuvant which potentiates the immunogenicity of the peptide. The liposomes are useful in vaccines.

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LIPOSOMES

Field of the Invention

The present invention relates to liposome preparations which combine small size with very high retention of added substances. The present invention also relates to liposomes containing synthetic peptides, e.g. for use in vaccines.

Background of the Invention

High efficiency entrapment (retention) of substances using minimal amounts of lipid is a prerequisite for the effective use of liposomes in a variety of applications. For example, a high entrapped drug to lipid mass ratio is necessary for an acceptable cost of formulation and an acceptable low risk of lipid-induced toxicity for pharmaceutical use. Unfortunately, current liposome preparations and methodologies giving high efficiency entrapment, as described by Szoka et al, Proc. Nat. Acad. Sci. USA 75 (1978) 4194-4198, and by Kirby and Gregoriadis, Biotechnology 11 (1984) 979-984, result in large liposomes, having mean diameters approaching 1 μm in size. This large size severely limits, for example, pharmaceutical use: such large liposomes, on intravenous injection, exhibit short half-lives in blood circulation; see Senior et al, Biochim. Biophys. Acta 839 (1985) 1-8. They are therefore unsuitable for maintaining increased concentrations of drugs and other substances within the vascular system. In addition, such large liposomes are unsuitable for use as delivery systems for targeting of drugs or other substances to specific sites, for example to diseased cells in the body.

Smaller liposomes, 200 nm or less in diameter, are considerably more stable, for example in the bloodstream, than the larger liposomes described above. Other features of smaller liposomes for pharmaceutical applications are that, unlike larger liposomes, they are able to reach the lymph nodes efficiently and to deliver vaccines and other agents to these sites: see Senior et al , loc. cit.; Turner

et al, Biochim. Biophys. Acta 760 (1983) 119-125; Allen et al, FEBS Letters 223 (1987) 42-46; Chow et al, Pharmacol. Exper. Ther. 248 (1989) 506-513; and Spanzer et al, Biochim. Biophys. Acta 836 (1986) 224-230. However, current techniques for preparing small liposomes, as described by Mayhew et al, Biochim. Biophys. Acta 775 (1984) 169-174, suffer from either a low efficiency of entrapment of substances or from a large lipid requirement (180 μmol per ml or greater) for efficient entrapment.

Most current vaccine preparations for infectious disease comprise either attenuated or killed infectious agents. Such preparations suffer from problems such as safety, for example reversion of attenuated to virulent strains, and lack of effectiveness, for example an incidence of non-responsive recipients. There is widespread interest in the possibility of synthetic peptide vaccines, especially against viral, bacterial and parisitic diseases (see Arnon, Synthetic Vaccines, 1987 (vols. 1 and 2), CRC Press Inc.) and, more recently, in cancer. The principle of a synthetic peptide vaccine is that the protective epitopes on, for example, infectious agents are copied as short synthetic peptides free of any infectious agent. In order to be effective, a synthetic peptide must possess a high level of immunogenicity and induce antibodies which cross-react extensively with the target organism or cell; see Van Regenmortel, Immunology Today 10 (1989) 266-272. In practice, peptides alone tend to possess weak immunogenicity, and anti-peptide antibodies often do not recognise the native protein antigen; see Jemmerson et al, Molecular Immunology 26 (1989) 301-307.

A common method for enhancing the immunogenicity of synthetic peptide epitopes is to combine the peptides with an immunological adjuvant. Adjuvants are agents which provoke potent immune responses to antigens, and include aluminium hydroxide (alum), saponins, pluronic polymers with mineral oil, killed mycobacterium in mineral oil (Freund's complete adjuvant), bacterial products such as

muramyl dipeptide, and immunogenic carrier proteins such as tetanus toxoid. Adjuvants, depending on their nature, induce humoural (HI) and/or cell-mediated immunity (CMI). However, most of such adjuvants are either unsuitable for combination with peptides, have toxic side-effect problems, or fail to induce both HI and CMI; see Gregoriadis, Immunology Today 11 (1990) 89-97. A further problem is interference of peptide immunogenicity by immune response to a carrier protein; see Etlinger et al, Science 249 (1990) 423-425.

As described elsewhere (see Gregoriadis, loc. cit.), liposomes have the potential for a safe and effective adjuvant for peptide vaccines, and the immunopotentiation of a number of synthetic peptides has been shown. However, such immunopotentiation by liposomes is not a general phenomenon. For example, liposomal immunopotentiation has not been observed with a pre-S peptide (amino acids 15-48) from Hepatitis B surface antigen (HBsAg) or with a 3VP2 peptide from poliovirus.

Summary of the Invention

Novel, small liposomes have a mean diameter less than 200 nm and high entrapment efficiency, and require low (<150 μmols per ml) concentrations of starting lipid. They are suitable for use in a variety of applications, including, for example, cosmetics for the sub-epidermal delivery of substances by liposomes, medical diagnostics for the stable entrapment of easily measurable substances, and pharmaceuticals (as described above).

Further, novel synthetic peptide vaccine preparations comprise liposomes containing entrapped or covalently-linked peptides together with molecules which augment the immune response to the peptides.

Description of the Invention

Examples of substances that may be entrapped in liposomes of the invention are pharmaceuticals, vaccines, genetic materials, enzymes, hormones, vitamins, carbohydrates, proteins/peptides, lipids, organic

molecules, and inorganic molecules or atoms. More specific examples are anti-tumour and anti-microbial agents, enzymes, hormones, vitamins, metal chelators and genetic material, preferably carbohydrates or proteins/peptides. The liposomes may be incorporated into vaccines.

The liposomes may be produced from one or more of phosphatidylcholine, cholesterol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, sphingomyelin or derivatives of these lipids, preferably phosphatidylcholine or cholesterol. The process for their preparation may be from a starting lipid concentration of less than 150 μmols per ml, to provide an efficiency of entrapment of >10% of a starting substance concentration. The method involves a combination of the dehydration-rehydration vesicle (DRV) process (see Kirby and Gregoriadis, loc. cit.) and the microfluidation process described by Talsma et al, Drug Development Ind. Pharm. 15 (1989) 197-207. Briefly, the method involves mixing lipids with substances to be entrapped and subjecting the mixture to controlled dehydration and rehydration to form large multilamellar vesicles with high entrapment efficiency. These large vesicles are then subject to microfluidation to produce small liposomes with 10-100% retention of the entrapped substances.

As will be apparent from the evidence given below, microfluidisation of solute-containing DRV's in PBS and the presence of unentrapped solute produces vesicles less than 200 nm in size, and which are suitable for the given purposes. Such vesicles retain, under the present conditions, 35-78% of the originally entrapped solute. Compared to procedures (Mayhew et al, loc. cit.) which employ much larger amounts of lipid to achieve efficient entrapment, the present approach provides preparations with augmented solute to lipid mass ratios and should also be more economical. The narrow distribution of final vesicle sizer must reflect the nature of the production process. Whereas sonication disrupts vesicles in a random mode,

producing a wide range of diameters, microfluidisation distorts large and flexible vesicles by extrusion through a capillary, thereby increasing the number of vesicles of similar size which "bud off" from simple parent vesicles.

While being applicable across the broad range of the invention, the minimum amount of retained solute is 35%, i.e. 2.5 mg with respect to a starting amount of 7.2 mg of the relevant substance. This is for up to 66 μmoles, i.e. up to 50 mg, phosphatidylcholine (approximate molecular weight 760) up to 25 mg cholesterol (molecular weight 387). Therefore, by means of the present invention, it is possible to produce liposomes having mean diameters of less than 200 nm which contain at least 50 mg of the entrapped substance per g phospholipid or at least 33 mg of the substance per g of liposome-forming materials. In Example 1, the liposomes retain approximately 35-78% of the originally entrapped test substances maltose or tetanus toxoid (herein referred to as solutes) following microfluidisation. This corresponds to an overall solute entrapment yield of >10%.

For example when including one or more adjuvants, liposome preparations are made by standard methods for producing liposomes, for example as described by Gregoriadis, ed., Liposome Technology (1984) vols. 1 to 3, CRC Press Inc. In such methods, liposome components, peptides and adjuvant molecules are either mixed or covalently linked prior to formation of liposomes.

The following Examples illustrate the invention, with reference to literature articles given above and also to Deamer and Uster, Liposome Preparation: Methods and mechanisms in: ed. Ostro, Liposomes, Marcel Dekker, Inc., New York, 1983, pp 27-51; Gregoriadis et al, Vaccine 5 (1987) 145-151; and Davis and Gregoriadis, Immunology 61 (1987) 229-234.

The source and grades of egg phosphatidylcholine (PC) and cholesterol have been reported elsewhere (Kirby and Gregoriadis, 1984). Maltose was from Sigma Chemical

Company (London) and immunopurified tetanus toxoid from Wellcome Biotechnology (Beckenham). D-[U-^{14}C]-maltose (20 GBq per μmol) was purchased from Amersham International (Amersham, U.K.) and toxoid-labelled (specific activity 5.43 mBq) with ^{125}I as described elsewhere (Gregoriadis et al, 1987).

The following abbreviations are used:

60 psi = 414 kPa

PBS - Phosphate-Buffered Saline

PC - Phosphatidylcholine

DRV - Dehydration-Rehydration Vesicles

SUV - Small Unilamellar Vesicles

dpm - disintegration per minute

Example 1

Preparation of liposomes

Solute-containing DRV liposomes were prepared by the procedure discussed elsewhere (Kirby and Gregoriadis, 1984; Gregoriadis et al, 1987). Briefly, small

unilamellar vesicles (SUV) made in distilled water from equimolar PC (16.5, 33, 66 or 132 μmol) and cholesterol were mixed with an equal volume (2.0 ml) of maltose (7.2 mg) or tetanus toxoid (7.2 mg) dissolved in PBS (0.44mM sodium phosphate, 2.7mM potassium chloride and 0.14 M sodium chloride, pH 7.4) and supplemented with traces (4.9×10^5 - 8.7×10^5 dpm) of the respective radiolabelled solutes. Following dehydration and controlled (Kirby and Gregoriadis, 1984) rehydration to form solute-containing DRV, the suspensions (2.0 ml) were each divided into two equal portions. One portion was centrifuged at 20,000g for 30 min, the pellet washed three times with PBS and suspended in 20 ml of the same buffer (washed DRV) for microfluidization. Entrapment of solutes was estimated by the assay (Kirby and Gregoriadis, 1984; Gregoriadis et al, 1987) of ^{14}C or ^{125}I radioactivity in the suspension, and results expressed as percentage of the solute originally present. The other portion (containing a mixture of entrapped and unentrapped solute) was also diluted in 20 ml PBS (unwashed DRV) and then microfluidized. In some experiments, PBS was replaced by distilled water in all steps involved in the entrapment of solutes in DRV and microfluidization of washed or unwashed preparations.

Microfluidization of liposomes

Washed and unwashed DRV (20 ml each) were passed for 1.8, 3.5, 5.2, 7.1 and 10.6 full cycles through a Microfluidizer 110™ kindly provided by Microfluidics Corp., Newton, MA, USA. The pressure gauge was set at 60 psi throughout the procedure to give a flow rate of 35 ml per minute. At the end of each of the 20 ml cycle intervals, samples containing maltose were dialysed exhaustively

against distilled water or PBS, and the toxoid containing vesicles or centrifuged for 30 min at 20,000g (1.8 and 3.5 cycles) or 35,000g (5.2, 7.1 and 10.6 cycles), the pellets washed twice in PBS or water and resuspended in 1.0 ml of the respective solvents. In the case of the toxoid, the supernatants obtained on centrifugation were passed (Senior et al, 1985) through Sepharose 4B columns; very little (less than 3%) of the solute in the centrifuged samples eluted in the liposome form. The extent to which washed and unwashed DRV's retained their solute content after microfluidization was estimated as follows: for washed DRV'S, radioactivity retained after dialysis or recovered in pellets after centrifugation was expressed as a percentage of the total amount in the sample before dialysis or centrifugation. Solute retention values thus obtained correspond to the percentage of the original entrapment values in the preparations before microfluidization. For unwashed DRV, the percentage of radioactivity retained after dialysis or centrifugation was expressed as % of the original entrapment values.

Measurement of vesicle size

Particle size distributions were measured by photon correlation spectroscopy of samples diluted in either water or PBS, using a Malvern Model 4700 apparatus (Malvern Instruments Ltd., Malvern, UK) equipped with a 25mW helium/neon laser. Mean diameters and size distributions are obtained: the z-average mean diameter, polydispersity factor and cumulative percentage mass and number undersize data were recorded as a function of the number of microfluidization cycles. The performance of the instrument was checked with monodisperse

polystyrene latex suspensions (Polysciences, UK) and mixtures of such latex samples to verify the ability of the system to accurately measure polydisperse or bidisperse systems.

Results and Discussion

The effect of DRV microfluidization on solute retention

As expected from previous work (Kirby and Gregoriadis, 1984; Gregoriadis et al 1987), entrapment of solutes in dehydration-rehydration liposomes was efficient, ranging from 19.2 to 66.1% of the starting material and dependent on the amount of phospholipid used (see legends to Figs 1-3). There was no significant difference in entrapment values in preparations made in distilled water or PBS (not shown). Retention values (quoted as a percentage of original entrapment values) by solute-containing DRV's microfluidized for up to 5.2 cycles in the presence of PBS are shown in Figs 1 and 2. It is apparent that retention values by both washed and unwashed DRV decrease or remain the same as the number of cycles increases (see also Fig. 3). Thus, values of 80-100% (maltose) after 1.8 cycles become 40-65% after 5.2 cycles (Fig. 1), whereas values for the toxoid (unwashed DRV) remain virtually unchanged (at around 75%) (Fig. 2). It is conceivable that during microfluidization, a process entailing breakage of the integrity of vesicle membrane and consequent leakage of entrapped solute occurs, large molecules such as proteins leak at slower rates than smaller molecules. Furthermore, no apparent difference in solute retention was observed for preparations containing varying amounts of PC (8.5-66 μmoles) (Figs. 1 and 2). Figs. 1 and 2 also indicate that, generally, a greater proportion of

solute (maltose or toxoid) is retained by unwashed vesicles (ie. preparations microfluidized in the presence of unentrapped solute) than by washed DRV. The presence of unentrapped solute during microfluidization diminishes solute leakage perhaps by reducing the osmotic rupture of the vesicles and by reducing initial concentration gradients across the membrane.

The mean diameter of microfluidized DRVs measured by dynamic light scattering after a given cycle interval depended on whether the DRVs were suspended in distilled water or PBS. We therefore attempted to determine the effect of the two media on solute retention during microfluidization. Results in Fig. 3 indicate that much more maltose is retained by DRVs in the presence of PBS and similar results (not shown) were obtained with toxoid-containing DRV's under identical conditions. Such an effect of PBS presence in the milieu could be attributed to its reduction of osmotic shock expected (Kirby and Gregoriadis, 1984) to occur on dilution of solute-containing DRV in hypotonic media.

The effect of microfluidization on vesicle size

The mean diameters of washed and unwashed maltose-containing DRV microfluidized in water or PBS (from experiment described in Fig. 3), measured by photon correlation spectroscopy, are presented in Table 1. They show that, in agreement with findings by others (Talsma et al, 1989), a smaller vesicle size was achieved for preparations processed in water than in PBS. Such differences in sizes, appearing after the 1.8 cycle interval, could be attributed (Deamer and Uster, 1983) to vesicle aggregation induced by the presence of salts in the PBS

medium, but also might be due to the increased lipophilicity of the lipids in the presence of electrolyte. Reduction in the size of all preparations tested was considerable after the initial 1.8 cycles interval. Subsequently, sizes levelled off to reach minimal values of 100-160 nm by 10.6 cycles depending on the medium (water or PBS) (it is of interest that a pattern similar to that of sizes in Table 1 was observed for solute retention with increasing cycle intervals (Fig.3), with values also levelling off after 3.5 cycles). Mean number diameter and mass diameter distribution data (Fig. 4 and Fig. 5) from the experiments recorded in Table 1 revealed a bimodal distribution of sizes in which there is an increasing number of small vesicles as the number of microfluidization cycles increases beyond the first few cycles; the mean number diameter of the sample, prepared in water (50mg maltose/water) is of the order of 300nm after 1.8 cycles, with a few vesicles with diameters up to 1μm in diameter. The larger diameter vesicles disappear rapidly on increasing cycling, so that after 10.6 cycles in water, a single narrow diameter (around 100 nm) distribution is obtained whereas in PBS the bimodal pattern is retained (Fig 4). However, the vesicle size distribution does not exhibit the gradual downward drift that occurs on, say, sonication but a transfer of sizes from one size band to another. In PBS, some vesicles undoubtedly flocculate so that there remains a percentage of vesicles with a high apparent diameter. Fig. 6 illustrates the distribution of diameters by mass. Here is clear evidence of the marked narrowing of the polydispersity of the vesicles. Coupled with the data from Fig. 4, this figure clearly shows the narrow distribution of sizes which is achieved after 10 cycles. Lidgate and coworkers' (1989) results show that in preparing parenteral emulsions of squalene by microfluidization, the particle size after 9-10 cycles is in the range of

0.07 to 0.2 μm which represent a much narrower distribution than other technologies.

Table 1 shows the average mean size (nm) of microfluidised DRV, i.e. maltose-containing washed or unwashed DRV (33 μmoles) PC were microfluidised in the presence of water or PBS for up to 10.6 cycles and samples measured for vesicle size (diameter in nm) by dynamic light scattering (Photon correlation spectroscopy). Polydispersity indexes ranging from 0.503 to 0.653 (water) and 0.517 to 0.653 (PBS) were similar to those obtained with some of the lipid compositions of liposomes employed by Talsma et al (1987).

Table 1. z - Average mean size (nm) of microfluidised DRV

DRV	Cycles 1.8	3.5	5.2	7.1	10.6
Washed					
Water	463.5	149.9	115.0	121.9	114.7
PBS	447.4	198.6	168.1	159.5	155.7
Unwashed					
Water	473.9	132.9	116.9	116.6	101.9
PBS	456.3	186.2	186.8	169.8	159.9

Description of the Drawings

Figure 1 shows entrapment values (on a scale of 0-100% of originally entrapped starting material) for ^{14}C-labelled maltose by DRVs following microfluidisation, with respect to cycles on the abscissa. ^{14}C-maltose containing DRVs composed of equimolar phosphatidylcholine (PC) and cholesterol were microfluidised in the washed (filled bars) or unwashed (shaded bars) forms in the presence of PBS for up to 5.2 cycles. Amounts of liposomal phospholipid passed through the microfluidiser were 8.25 (A), 16.5 (B), 33 (C) and 66 (D) μmols. Values denote % retention of the originally entrapped ^{14}C-maltose. Original entrapment values were 27.1 (A), 33.6 (B), 66.1 (C) and 65.7 (D).

Figure 2 shows entrapment values for ^{125}I-labelled tetanus toxoid by DRVs following microfluidisation. Details are as in Figure 1 except that amounts of liposomal phospholipid passed through the microfluidiser were 8.25 (A) and 33 (B) μmols and original entrapment values were 19.2 (A) and 56 (B).

Figure 3 shows the effect of the liquid medium on ^{14}C-maltose retention by DRVs during microfluidisation. Details are as in Figure 1 except that DRVs were microfluidised in the presence of distilled water (A) or PBS (B) for up to 10.6 cycles, the amount of liposomal phospholipid passed through the microfluidiser was 33 μmols and original entrapment values were 54.3 (A) and 55.2 (B).

Figure 4 shows the size distribution (as a percentage number) of vesicles containing maltose prepared in the presence of water as a function of the number of cycles (Δ = 10.6 cycles; ▪ = 3.5 cycles; O = 1.8 cycles) through the microfluidiser, showing progression to a mean distribution with a mean diameter (on the abscissa) of approximately 100 nm after 10.6 cycles.

Figure 5 is on the same basis as Fig. 4. It shows the size distribution of vesicles containing maltose prepared in the presence of PBS (50 mg maltose/PBS) as a function of the number of cycles through the microfluidiser, showing

that, after 10.6 cycles, the number of vesicles with diameters greater than 300 nm is decreased but a biphasic distribution is still maintained.

Figure 6 shows the mass distribution of vesicles containing maltose (50 mg maltose/water) as a function of cycles through the microfluidiser as in Figure 4, showing the narrow distribution size after 10.6 cycles.

Example 2

This Example shows that interleukin-2 (IL-2) acts as an effective adjuvant for the poliovirus 3-VP2 peptide when entrapped with the peptide in the same liposome. Balb/c mice were injected intramuscularly in groups of five on days 0 and 28 with either 5 μg free 3-VP2 peptide, or with liposomes containing 5 μg peptide, or with liposomes containing 5 μg peptide and 7000 Cetus units of IL-2 (recombinant mutein IL-2, obtained from Cetus). Liposomes were formed from equimolar distearoyl phosphatidylcholine and cholesterol using the dehydration-rehydration procedure (see Kirby and Gregoriadis, 1984). Ten days following the second injection, serum IgG antibodies specific for 3-VP2 peptide were estimated by ELISA assay (see Davis and Gregoriadis, 1987) whereby microtitre wells were coated with 3-VP2 peptide, diluted sera samples (1:1280 for IgG1, 1:160 for other subclasses) were added, and bound IgG's were measured through binding of IgG subclass specific antibodies. The ELISA signal measured as optical density at 492 nm was produced using horseradish peroxidase-labelled second-antibody and 3,3',5,5'-tetramethylbenzidine (Miles) as substrate. Table 2 gives the results of the IgG subclass ELISA (median values underlined). Comparison by Kruskall-Wallis statistical analysis of values for groups of animals receiving liposomal 3-VP2 and liposomal 3-VP2/IL-2 revealed a significant elevation of IgG levels due to the effect of IL-2 for all IgG subclasses except IgG2b.

Example 3

SJL H-2' mice were injected intramuscularly in groups of five on days 0 and 28 with either 20 μg free pre-S

peptide or with liposomes containing 20 μg pre-S peptide or with liposomes containing 20 μg S peptide and 20 μg pre-S peptide. Liposomes were formed from equimolar egg phosphatidylcholine and cholesterol using the dehydration-rehydration procedure (see Kirby and Gregoriadis, 1984). Ten days following the second injection, serum IgG antibodies specific for pre-S peptide were estimated by ELISA assay (see Davis and Gregoriadis, 1987), whereby microtitre wells were coated with pre-S peptide, diluted samples (1:640) were added, and bound IgG_1 was measured through binding of IgG_1 specific antibodies. The ELISA signal measured as optical density at 492 nm was produced using horseradish peroxidase-labelled second-antibody and 3,3',5,5' tetramethylbenzidine (Miles) as substrate. Table 3 gives the results of the IgG_1 subclass by ELISA (median values underlined).

Statistical analysing by Kruskall-Wallis revealed highly significant increase in IgGI response to pre-S peptide for liposomes with co-entrapped pre-S and S peptides.

Peptide formulation	Secondary immune response			
	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3
Free 3VP2	0.086	0.074	0.075	0.125
	0.092	0.085	0.079	0.128
	0.095	0.086	0.080	0.135
	0.096	0.113	0.080	0.141
	0.099	0.180	0.084	0.149
Liposomal 3VP2	0.110	0.100	0.079	0.151
	0.112	0.107	0.085	0.161
	0.123	0.124	0.093	0.185
	0.130	0.143	0.096	0.206
	0.181	0.144	0.096	0.277
Liposomal 3VP2 + IL-2	0.101	0.101	0.070	0.153
	0.285	0.143	0.075	0.269
	0.341	0.334	0.076	0.323
	0.448	0.492	0.104	0.359
	1.021	0.935	0.142	0.499
Kruskall-Wallis (one-way)	H=10.5 $p<0.009$	H=5.95 $0.01<p<0.049$	H=3.64 $p>0.102$	H=10.5 $p<0.009$

TABLE 2

Secondary immune response to pre-S peptide

Peptide formulation	IgG_1
Free pre-S peptide	0.080
	0.090
	0.095
	0.100
	0.100
Liposomal pre-S peptide	0.075
	0.080
	0.085
	0.090
	0.110
Liposomal pre-S and S peptides	0.610
	0.680
	0.780
	0.850
	0.900

TABLE 3

CLAIMS

1. Liposomes having a mean diameter not exceeding 200 nm and containing at least 33 mg of one or more entrapped substances per g liposome-forming material.

2. Liposomes according to claim 1, wherein the one or more substances is covalently linked to a lipid component of the liposome.

3. Liposomes according to either preceding claim, wherein the lipid component is one or more of phosphatidylcholine, cholesterol, phosphatidylglycerol, phosphatidyl-ethanolamine, phosphatidylserine, phosphatidic acid, sphingomyelin or derivatives of these lipids.

4. Liposomes according to claim 3, wherein the lipid components are phosphatidylcholine and cholesterol.

5. Liposomes according to any preceding claim, wherein the one or more substances are selected from carbohydrates and peptides.

6. Liposomes comprising, co-entrapped or covalently linked in the same liposome, a synthetic peptide and an adjuvant which potentiates the immunogenicity of the peptide.

7. Liposomes according to claim 6, wherein the adjuvant is recognised by or interacts with T cells.

8. Liposomes according to claim 6, wherein the adjuvant is a peptide having a T cell recognition site.

9. Liposomes according to claim 6, wherein the adjuvant is IL-2.

10. Liposomes according to claim 6, wherein the adjuvant comprises a peptide having a T cell recognition site and IL-2.

11. Liposomes according to any of claims 5 to 10, wherein the peptide includes all or part of amino-acid residues 110-137 of Hepatitis B surface antigen.

12. Liposomes according to any preceding claim, for therapeutic use.

13. A vaccine composition comprising liposomes according to any preceding claim and a physiologically-acceptable excipient.

14. Use of liposomes according to any of claims 5 to 11, for the manufacture of a medicament for use in eliciting an immunogenic response to the synthetic peptide.

15. A method for preparing liposomes having a mean diameter not exceeding 200 nm and containing one or more entrapped substances in an amount exceeding 10% of starting amounts.

16. A method for producing liposomes having a mean diameter not exceeding 200 nm, comprising:

(a) subjecting a mixture of lipid components and substances to be entrapped or covalently linked to the lipid component, to dehydration and controlled rehydration, to give large multilamellar vesicles; and

(b) subjecting the large multilamellar vesicles to microfluidisation.

17. A method according to claim 15 or claim 16, wherein the starting concentration of lipid is no more than 100 μmols per ml.

18. Liposomes according to any of claims 15 to 17, wherein the total starting concentration of all substances is no more than 10 mg per ml.

19. A method according to any of claims 15 to 18, for preparing liposomes having the characteristics of any of claims 1 to 11.

FIGURE 1

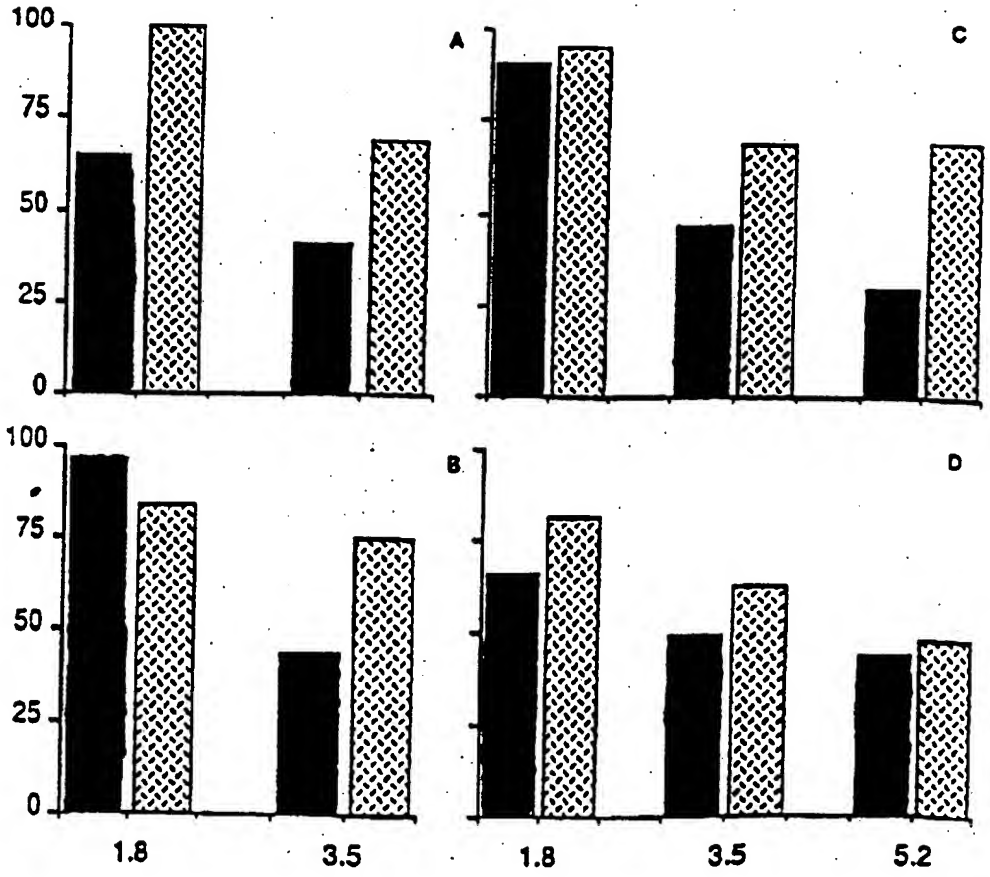


FIGURE 2

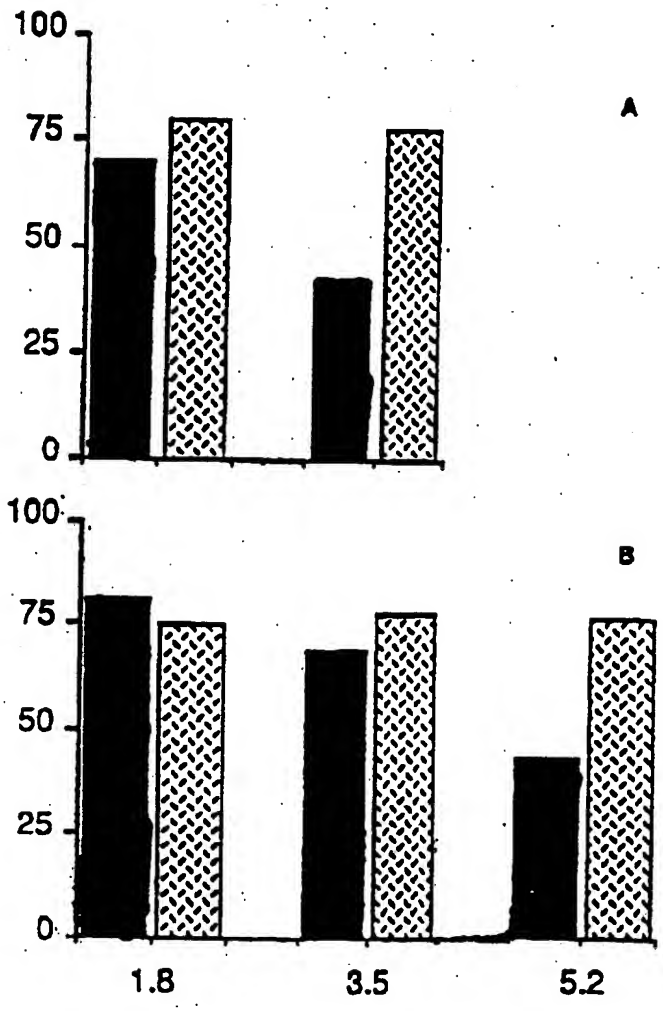


FIGURE 3

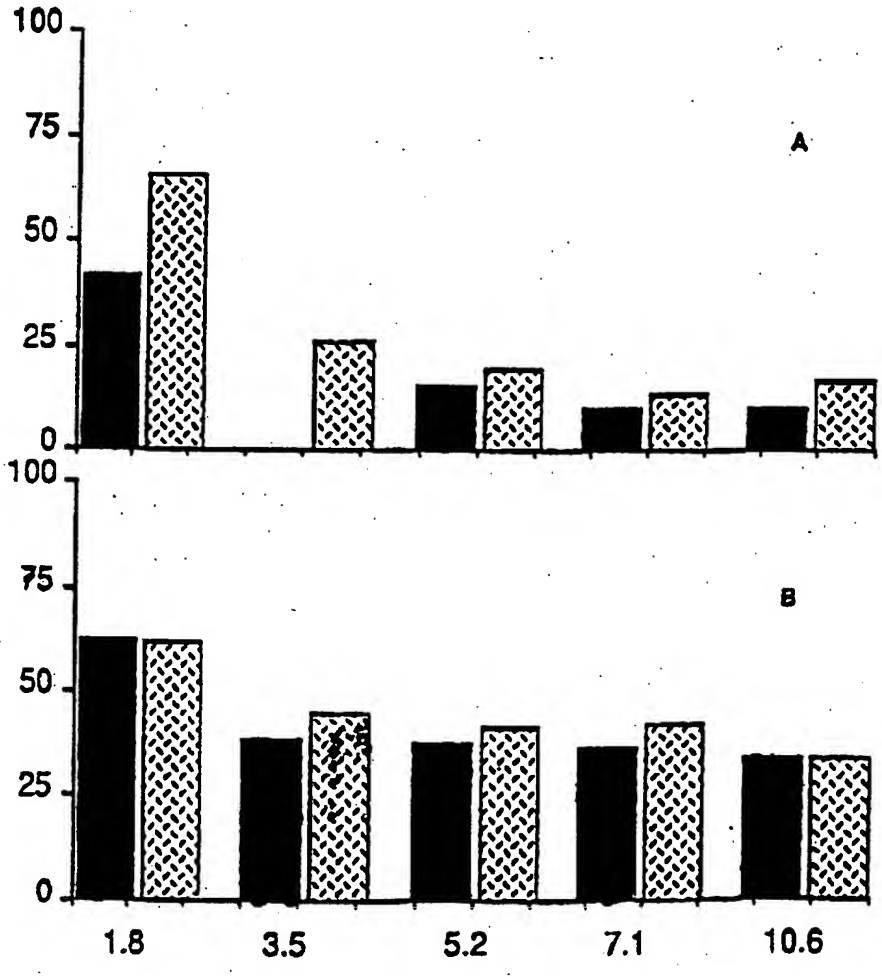


FIGURE 4

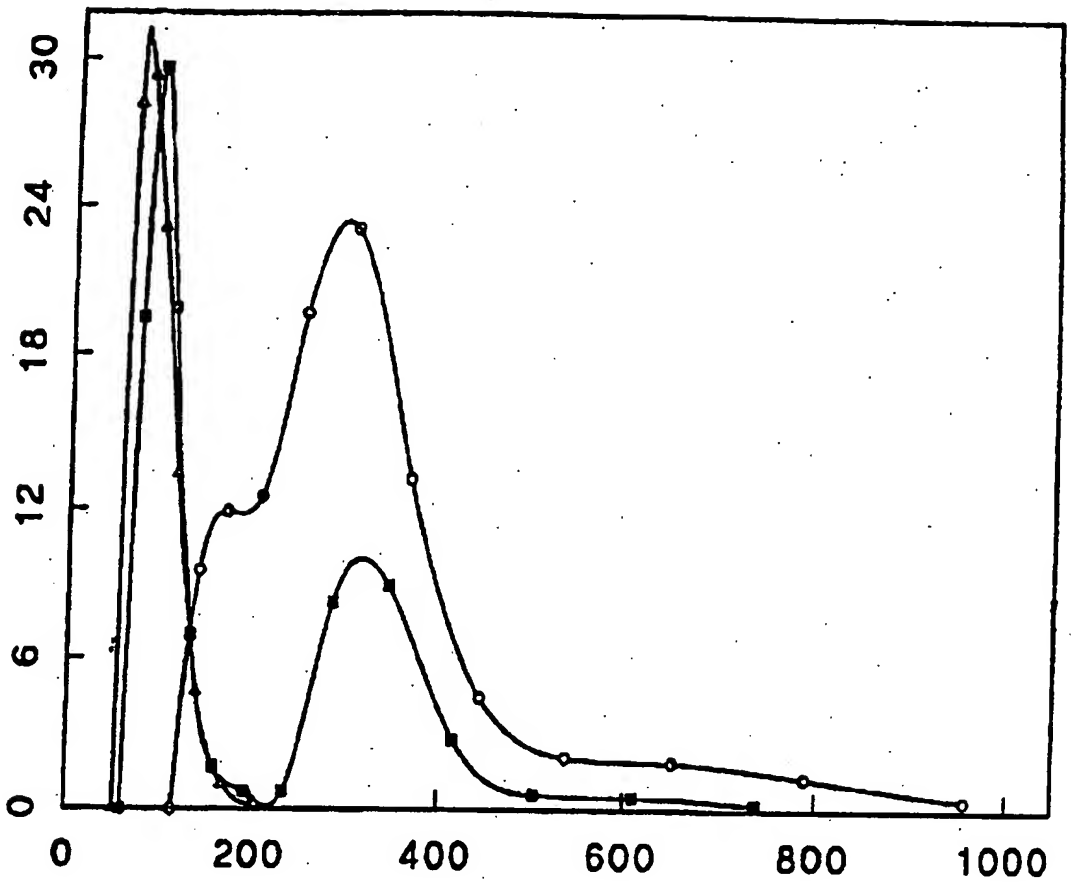


FIGURE 5

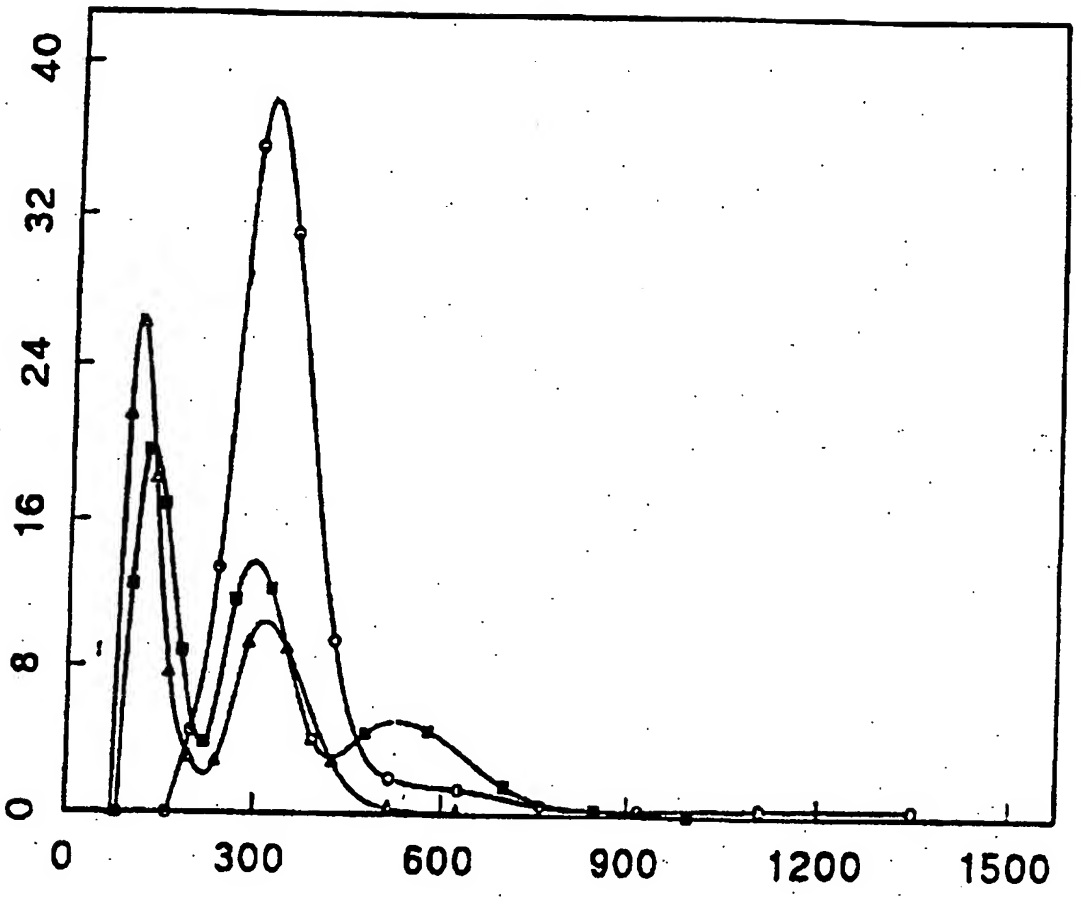
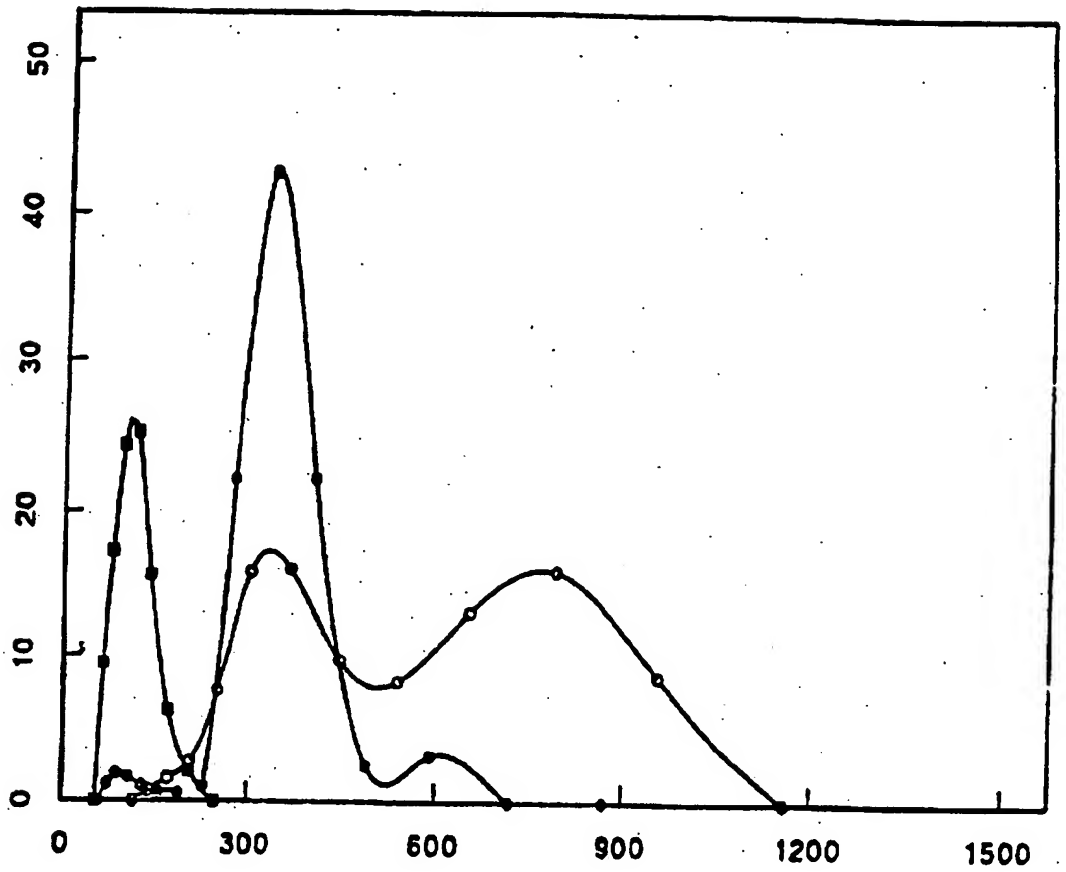


FIGURE 6



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01547

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K9/127

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X Y	EP,A,0 290 296 (THE LIPOSOME COMPANY) 9 November 1988	1,3,4,12 2,5-11, 13,14
Y	VACCINE vol. 5, July 1987, GUILDFORD pages 145 - 151; G.GREGORIADIS ET AL.: 'liposomes as immunological adjuvants: antigen incorporation studies' see page 148, col.2, second paragraph (document cited in the application)	2
Y	FR,A,2 624 741 (INSTITUT PASTEUR) 23 June 1989 see page 5 - page 6; examples 1,6	5-10,13, 14

-/--

* Special categories of cited documents :[10]

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 JANUARY 1992	30 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	BENZ K.F. Benz

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,9 004 412 (REGENTS OF THE UNIVERSITY OF MINNESOTA) 3 May 1990 see page 5, line 16 - line 31 see page 11, line 26 - page 13, line 22 see page 31 - page 32; example 6	11
Y	US,A,4 235 877 (FULLERTON) 25 November 1980 see column 3 - column 4; examples 3,5	11
A	WO,A,9 004 943 (E. UNGER ET AL) 17 May 1990 see page 48 - page 51; examples 8,9	15-19

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9101547 SA 51307

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 14/01/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		EP-A-	0440742	14-08-91
		CA-A-	2001737	19-01-91
US-A-4235877	25-11-80	None		
WO-A-9004943	17-05-90	EP-A-	0442962	28-08-91